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Synthetic decapeptide reduces bacterial load and accelerates healing in the wounds of restraint-stressed mice

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ABSTRACT

Wound healing is a complex process involving four transitional yet concurrent stages: coagulation, inflammation, cell proliferation/epithelialization and remodeling. These overlapping stages occur uneventfully in normal physiology. However, during psychological stress, the inflammatory response can become dysregulated and result in increased susceptibility to bacterial infection and delayed wound closure. In our restraint stress model, cutaneous wounds of stressed SKH 1 mice demonstrate signifi cantly higher levels of bacterial load, and healing progresses at a rate 30% slower, than in non stressed mice. The purpose of this study was to test the hypothesis that a synthetic antimicrobial decapeptide (KSLW) enhances bacterial clearance during stress impaired healing in mice. Here, using a Pluronic block copolymer nanocarrier, we endeavored to identify an efficient drug delivery system for KSLW, which would enhance the stability, substantivity and function of the cationic peptide in delayed healing wounds. In this study, intradermal treatment of excisional wounds of stressed mice with 2 mg/ml KSLW loaded in Pluronic F68, resulted in a sustained antimicrobial effect through post operative day 5, with a 2 log ($p < 0.01$) reduction in bacterial load compared with other stressed mice. The demonstrated bacte rial reduction in KSLW treated stressed mice did not approach the levels observed among control mice. Furthermore, treatment of stressed mice with KSLW improved healing, resulting in significantly faster ($p < 0.05$) wound closure from days 2 to 5 post wounding, relative to untreated stressed mice and stressed mice treated with Pluronic alone. These findings suggest that Pluronic F68 is an efficient carrier for KSLW, which improves its stability and activity in impaired dermal wounds.

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1. Introduction

Wound healing is a complex process involving four transitional yet concurrent stages: coagulation, inflammation, cell proliferation and epithelialization and remodeling (Bjarnsholt et al., 2008). These overlapping stages occur uneventfully in normal physiology. However, during psychological stress, the inflammatory response can become dysregulated and result in increased susceptibility to bacterial infection (Rojas et al., 2002) and delayed wound closure (Kiecolt Glaser et al., 1995; Marucha et al., 1998; Padgett et al., 1998).

Toll like receptors represent an ancient and evolutionarily conserved receptor family, which recognize conserved pathogen associated molecular patterns shared by large groups of microorgan isms (Aderem and Ulevitch, 2000; Kaisho and Akira, 2001; Zhang et al., 2008). Stimulation of Toll like receptors (TLRs) leads to the in nate induction of phagocyte chemoattractant molecules/proinflam matory cytokines, and the activation of genes necessary for initiating adaptive immune responses (Medzhitov et al., 1997). TLR 4 is an LPS receptor (Sodhi et al., 2007), and on binding LPS transfers the signal into the cell, resulting in neutrophil oxidative burst (Remer et al., 2003) and macrophage activation (Wang et al., 2008). Previous studies suggest a role for TLR 4 in mediating stress induced im mune suppression and splenic lymphocyte apoptosis, in mice sub jected to restraint stress (Zhang et al., 2008). Normal healing wounds, populated by opportunistic bacteria, will display in creased levels of TLR 4, as well as TLR 2, in response to microbial components, including LPS, cell wall glycolipids cell wall glycolip ids, lipoarabinomannan and mannosylated phosphatidylinositol (Jones et al., 2001), correlating with macrophage activity. This work investigated the expression pattern of TLR 4 in wounded skin of stressed mice, demonstrating suppressed immunologic activity.

Within the last two decades, antimicrobial peptides have re ceived much attention as potential therapeutic agents. Hong and co workers (1998), by using combinatorial peptide libraries,

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identified an activity optimized decapeptide (KSL, KKVVFKVKFK NH_2) which exhibited a broad spectrum of activity against bacteria and fungi without hemolytic activity. *In vitro* efficacy studies of the decapeptide revealed that a minimal inhibitory concentration (MIC) against selected human oral bacteria ranged from 3 to 100 μg/ml, and *in vitro* toxicity studies showed that KSL, at concen trations up to 1 mg/ml, did not induce cell death or compromise the membrane integrity of human gingival fibroblasts (Concannon et al., 2003). NMR analysis confirmed that KSL adopts an α helical structure in DMSO, that is maintained by six intra molecular hydrogen bonds (Concannon et al., 2003). Other structural studies, performed by circular dichroism spectroscopy, indicated β sheet structure of the peptide in lipid membranes, required for perme abilization (Choi et al., 2004). KSLW (MW 1308), is an analog of the parent KSL molecule, in which the Lys^6 residue is replaced with Trp. This substitution resulted in increased peptide stability to hydrolytic enzymes, while preserving its potent antibacterial activ ity against several oral species (Na et al., 2007). Based on findings from these *in vitro* studies, we became interested in testing the ef fect of KSLW in our murine stress impaired wound healing model.

The search for the optimal antimicrobial agent, which perhaps enhances bacterial clearance, in a highly potent and sustained manner, as well as improving host cell function, remains a goal of wound care specialists. Multiple delivery systems, with sus tained release properties, and the potential for reducing exposure of drugs to hydrolytic enzymes, have been employed in wounds to increase the duration of bioactivity of therapeutic agents (Ovington, 2007). Surfactant micelles have been shown to enhance the bioavailability for not only lipophilic drugs (which presumably possess some affinity for the micelle's hydrophobic core) but also for polar drugs including poorly absorbable polar compounds and macromolecules, such as peptides and proteins (Torchilin, 2002). Pluronic block copolymers contain two hydrophilic ethylene oxide blocks and a hydrophobic propylene oxide block, arranged in a ba sic A B A structure (Kabanov et al., 2002). Like PEGylated block polymers, the Pluronics have the capacity for self assembly into micelles above a low critical micellar concentration (CMC) (Kabanov et al., 2002). They are high molecular weight, non ionic, surface active agents with broad pharmaceutical and medical applications (Hadgraft and Howard, 1982; Hokett et al., 2000), and have been used extensively in the delivery of low molecular mass drugs and polypeptides (Kabanov et al., 2002).

The purpose of this study was to test the hypothesis that the synthetic decapeptide KSLW enhances bacterial clearance during stress impaired healing in mice. Here, using a Pluronic block copolymer as a nanocarrier, we endeavored to identify an efficient drug delivery system for KSLW, which would enhance the stability, substantivity and function of the cationic antimicrobial peptide in excisional wounds.

2. Materials and methods

2.1. Animal selection

For all animal experiments in this study, we selected the SKH 1e mouse strain (Skh: hairless 1). This mouse is euthymic and immunocompetent, and has been used widely in wound healing models, dermal research/photosensitivity studies, and safety and efficacy testing. This protocol was approved by the Committee on Animal Research at the University of Illinois at Chicago. Virus anti body free, female mice approximately 9 10 weeks of age were purchased from Charles River Laboratories International, Inc. (Wilmington, MA). Mice were housed in conventional cages, 4 5 per cage, in the UIC College of Dentistry's animal facility under a 12:12 light:dark cycle with lights on at 06:00 h. Water and food were available *ad libitum*. Animals were allowed 1 2 weeks to acclimate to the vivarium.

2.2. Restraint stress paradigm

We used the restraint stress paradigm to induce stress in ran domly assigned mouse groups. This model provides a consistent, physiologic and psychological stress response (Padgett et al., 1998). Each mouse subjected to restraint was placed in a separate well ventilated 50 ml centrifuge tube for eight cycles in total: three times prior to and five times following wounding. On each of these days, the mice were placed in tubes at 1800 h, and removed by 06:00 h (12 h duration). The duration of this paradigm is necessary to break the circadian rhythm of stress hormones. Since animals in the restraint tubes do not have access to food and water, control mice were deprived of food and water during the same 12 h period, but allowed to roam free in their cages. All animals were weighed on a daily basis, starting prior to the first period of restraint (RST) or food water deprivation (FWD = control).

2.3. Anesthesia and wounding

All mice were anesthetized with 250 μl doses of a Ketamine Xylazine saline cocktail (ratio 4:1:35) consisting of Ketamine 100 mg/kg and Xylazine 5 mg/kg, administered intra peritoneally. The dorsal skin was cleaned with isopropanol and two uniform, bilateral, full thickness wounds were created, just below the shoulder blades, using a sterile 3.5 mm biopsy punch (Miltex Inc., York, PA).

2.4. Synthesis of KSLW antimicrobial peptide

The antimicrobial peptide, KSLW (KKVVFWVKFK NH_2), was synthesized by standard solid phase procedures as described by Hong et al. (1998), by using 9 fluorenylmethoxy carbonyl (Fmoc) chemistry on a model 90 automatic peptide synthesizer (Advanced ChemTech), and purified by reverse phase HPLC (series 1100; Hewlett Packard) on a Vydac C18 column. Peptide purity was con firmed by MALDI TOF (matrix assisted laser desorption/ ioniza tion time of flight) MS, as performed by the laboratories of AnaSpec, with the molecular mass found at 1308.3.

2.5. Fluorescently labeled KSLW

Fluorophore labeled KSLW peptide was prepared by AnaSpec, using HiLyte Fluor™ 488, with >95% HPLC purity. HiLyte Fluor™ 488 amine is a carbonyl reactive fluorescent labeling dye, and fluorescence is independent of pH from 4 to 10 (AnaSpec, Inc.). Total mass of the conjugated peptide (HiLyteFluor™488 LC KKVVFWVKFL NH_2) was found at 1778.5.

2.6. Preparation of KSLW Pluronic F68 solution

A micelle forming block copolymer Pluronic® F68 [MW: 8400 (BASF Corporation, Mount Olive, NJ)], was diluted in sterile molec ular grade water to a concentration of 12 mM. A stock solution of KSLW peptide was filter sterilized and mixed with polymer solu tion, at a concentration of 2 mg/ml, via pipetting. The KSLW Plu ronic solution was incubated for 1 h, at 37 °C, collected in 1 ml syringes (Becton Dickinson, Franklin Lakes, NJ), and maintained at room temperature until wound treatment.

2.7. Topical application of antimicrobial peptide

KSLW preparations and normal saline were applied topically and bilaterally to wounds of RST and FWD mice, at the time of

surgery, in 20 μl doses. The purpose of the early application of the treatment agents, at the time of surgery, was to strategically re duce the potential of bacterial contamination in the wounds of stress impaired animals.

2.8. Intradermal drug delivery

For intradermal delivery experiments, KSLW solutions at a con centration of 2 mg/ml were prepared in 12 mM Pluronic F68 block copolymer (F68). Introduction of KSLW peptide solution or empty control vehicle (F68) was achieved via local injection of 50 μl solu tion in the tissue surrounding the wounds, with the help of Micro Fine IV syringes 28G1/2 (Becton Dickinson). Careful wound manipulation ensured no direct disruption of the wound site. An additional 10 μl of solution were applied directly to the wounds.

2.9. Measurement of wound infection

Wound infection was assessed with measurement of bacterial load at the wound site by methods adapted from Rojas et al. (2002). To harvest healing wounds for bacterial assays, mice were anesthetized (as described above) and wounds were harvested on postoperative days 1 and 5, via 6.0 mm punch biopsy, and homog enized in 1 ml of sterile 1X PBS, using a Tissue Tearor Homogenizer (Cole Parmer, Vernon Hills, IL). Serial dilutions (1:10) were plated, in duplicate, on brain heart infusion agar (Becton Dickinson), incubated for 24 h at 37 °C, and quantified by counting the number of colonies formed.

2.10. Determination of peptide structure in Pluronic solution

Circular dichroism spectra of KSLW and controls were mea sured on a Jasco J 810 CD spectropolarimeter (Tokyo, Japan), using a quartz cell 1 mm path length between 190 and 260 nm at room temperature and 1.0 nm band width. Trifluoroethanol (TFE) was obtained from Aldrich (Milwaukee, WI). The concentration of KSLW peptide was 2 mg/ml in 12 mM Pluronic solution (pH 5.0) or 50% TFE (v/v). Heights were determined with a specificity of 2 mdeg, and expressed as $[\theta] \times 10^{3}$, deg cm^2/d mol (Saxena and Wetlaufer, 1971). The α helicities were determined from the mean residue ellipticity $[\theta]$ at 222 nm, according to the relation $[\theta]$ 222 = 30300 $[a]$ 2340, where $[a]$ is the amount of helix (Chen et al., 1972; Oh et al., 1998).

2.11. Determination of minimum inhibitory and minimum bactericidal concentrations

In vitro bacterial assays were performed, using 96 well microti tre plates. The type strain of *Staphylococcus epidermidis* (ATCC 14990, Manassas, VA), was grown to stationary phase in Mueller Hinton Broth (MHB) and diluted with fresh MHB to an OD_{600} value of 0.01. Microtitre plates were prepared using sterile molecular grade water as a diluent for treatment solutions. KSLW solutions were prepared in normal saline and F68 solution at starting con centrations of 1000 μg/ml, or sterile water at a starting concentra tion of 200 μg/ml, followed by serial 1:2 dilutions to 0.03 μg/ml. A 500 μl aliquot of a diluted cell suspension was pipetted into each well of the sterile plates, incubated (under aerobic conditions) at 37 °C for 24 h, and read spectrophotometrically at OD_{600}. Control wells contained sterile media, undiluted bacteria control, and empty wells. Purity of bacterial cultures and detectable MIC/MBC optical density readings were confirmed on 5% Sheep blood agar plates. All bacterial assays were performed in triplicate.

2.12. Release kinetics of KSLW peptide in Pluronic F68

Measurements of the degree of drug release are based on de creases in fluorescence intensity, when Hilyte Fluor™ 488 labeled KSLW is transferred from the hydrophobic environment of micelle cores to the aqueous environment. Release kinetics experiments were performed with a rapid equilibrium dialysis plate (Pierce Bio technology, Rockford, IL). Equilibrium dialysis inserts consisted of side by side chambers separated by an O ring sealed vertical cyl inder of dialysis membrane with a molecular weight cutoff of 8000. Fluorescently labeled KSLW peptide was incubated for 1 h at 37 °C in 12 mM F68 solution, at a concentration of 2 mg/ml, spontaneously partitioning into the core of Pluronic micelles (Mar in et al., 2002). For each replicate, a 200 μl aliquot of KSLW F68 solution was added to the sample chamber and 350 μl 1× phos phate buffered saline was added to the dialysis buffer chamber. The plate was covered and incubated on an orbital shaker at approximately 100 rpm. To determine the amount of released pep tide, at specific time points after micellization, 100 μl KSLW + F68 solution was collected and transferred to a black, clear bottom 96 well plate, and fluorescence intensity (excitation 488/emission 530) was read using a Spectra Gemini XS spectrophotometer (Molecular Devices, Sunnyvale, CA). Fresh dialysis buffer was added to buffer chamber every hour, until respective time point measurements were completed. A standard curve with known con centrations of fluorescent KSLW in 100 μl of 12 mM F68 was pre pared per reading, and used for quantitative comparisons of each sample. All drug release experiments were performed in triplicate. Data were collected and analyzed using SOFTmax® PRO software (Molecular Devices).

2.13. Measurement of wound size

Beginning on the day of wounding (day 0), each animal's wounds were photographed daily until specific time points post wounding, or complete closure was observed. Digitized photo graphs of each wound were taken with a standard sized dot placed next to the site and analyzed by photoplanimetry (Marucha et al., 1998). Wound size was expressed as the percentage of the wound area determined on every post wounding day, compared with the original wound area.

2.14. Total RNA isolation

After wounding (day 0) and biopsy (days 1 and 5) harvested tis sue samples were immediately stored in TRIzol® (Invitrogen) and frozen at 80 °C. Upon processing, samples were homogenized with a Tissue Tearor homogenizer (Cole Parmer), and total RNA was extracted through chloroform extraction protocol, RNA precipita tion in isopropanol, RNA washing with 75% ethanol and dissolving in 20 μl of DEPC treated water. Spectrophotometry of RNA samples was done at absorbance wavelengths of 260 and 280 nm. A260/280 ratios were used to calculate the total RNA concentration and purity.

2.15. Reverse transcription

Synthesis of cDNA was completed using SuperScript™ First Strand Synthesis System for RT PCR (Invitrogen). One microgram total RNA was added to 2 μl of random hexamers (50 ng/μl) and 1 μl of 10 mM dNTPs and incubated at 65 °C for 5 min in a Gene Amp® PCR System 2700 (Applied Biosystems). A mixture of 2 μl of 10× RT buffer, 4 μl of 25 mM $MgCl_2$, 2 μl of 0.1 M DTT and 1 μl of recombinant ribonuclease inhibitor was added to each reac tion tube, briefly vortexed, centrifuged, and incubated at 25 °C for 2 min. Then, 1 μl of SuperScript™ II RT (reverse transcriptase) was added to the tubes and thermocycled at 25 °C for 10 min, 42 °C for

50 min, and 70 °C for 15 min. cDNA synthesis was completed after digestion with 1 μl of RNase H for 37 °C for 20 min to remove residual RNA from the cDNA:RNA hybrid.

2.16. Real time reverse transcription PCR analysis

Amplification of target cDNA was accomplished using the ABI Prism 7000 Sequence Detection System. Using Primer Express software (PE Biosystems) real time probe and primers for mouse TLR 4 (Table 1). Two microliters of cDNA diluted 1:10 were added to 12.5 μl of PE Master Mix (PE Biosystems), 2.5 μl target gene probe primer mix, 5.5 μl of DEPC treated water, and 2.5 μl of GAPDH probe and primer mix. The relative amount of target cDNA in each sample was determined by measuring fluorescence of the probe specific for each gene and determining the ΔRn/Ct.

2.17. Data analysis

All statistics were performed using SPSS 15.0 (Chicago, IL). Main effect statistical significance was determined at $p < 0.05$. Student's *t* test and One Way ANOVAs were performed with 'colony count' as the dependent variable and "stress/no stress" and "treatment group" as the Between Subjects factors. Wound closure data were analyzed via Repeated Measure ANOVAs, performed with Bonferroni post hoc analyses for all statistically significant main effects. These data are presented as the mean and standard errors. Gene expression data were calculated and presented as the relative difference in mRNA levels compared to control. The values generated for each sample were adjusted by the corresponding amount of housekeeping gene (GAPDH) mRNA expression.

3. Results

3.1. Circular dichroism spectropolarimetry

The proposed mechanism for antimicrobial activity of KSLW is based on its ability to assume secondary structure on association with bacterial membrane. The CD spectra were obtained from KSLW (at concentration 2 mg/ml) in 50% TFE and 12 mM F68 solution. The two sets of spectra appeared similar for alpha helicity. Although amplitudes varied, examination the α helix spectra were found to be quantitatively similar, with the positioning at 198, 209, and 215 nm in TFE, and 195, 203, 207 nm in F68. Both plots are consistent with models demonstrating alpha helicity in peptide secondary structure (graphs not shown).

3.2. Bacterial load following topical treatment with KSLW

Using our murine wound healing model, we evaluated the antimicrobial effect of topical administration of two predetermined concentrations of KSLW dissolved in saline: 2 mg/ml (K2) and 0.5 mg/ml (KLOW). This study demonstrated a significant reduction (1 log) in bacterial counts among RST mice treated with K2 on day 1 post wounding (data not shown). However, no significant differences, in bacterial load, were observed on day 5, among any restraint stressed mice groups (data not shown).

Table 1
Selected probes and primers.

Selected probes and primers		
Mouse TLR-4	Probe	5′-CTATAGCATGGACCTTACCGGG-3′
Mouse TLR-4	Forward primer	5′-CATGGAACACATGGCTGCTAA-3′
Mouse TLR-4	Reverse primer	5′-GTAATTCATACCCCTGGAAAGGA-3′
GAPDH	Probe and primers	Proprietary

3.3. Intradermal injection of KSLW in Pluronic F68

The intradermal route for drug delivery appeared to be promising, in our wound healing model. In this study, restraint stressed mice treated via intradermal delivery of either 1 mg/ml KSLW (K1) or 2 mg/ml KSLW (K2), in F68 solution, demonstrated a significant decrease in ($p < 0.01$) bacterial load on day 1 post wounding, compared with other RST mice (Fig. 1). In addition, no significant differences ($p = 0.77$) were observed between RST mice receiving K2 and FWD control animals at this early time point. The antimicrobial effect of high dose KSLW (K2) delivered in F68 block copolymer was sustained through day 5 post wounding, with a greater than 2 log ($p < 0.01$) reduction in bacterial load among RST mice (Fig. 1). However, the amount of bacterial reduction (on day 5) did not approach the levels observed in FWD control animals. Indeed, there was a significant difference ($p = 0.028$) in wound bacteria counts of RST mice treated with K2, compared with FWD (Fig. 1). Stressed mice treated with empty F68 carrier [(RST + F68) without peptide] displayed no differences from untreated RST mice on days 1 or 5.

3.4. In vitro assays (MIC/MBC: OD_{600} absorbance and 5% Sheep blood agar plates)

We conducted *in vitro* bacterial assays against *S. epidermidis* to determine the minimum inhibitory and minimum bactericidal concentrations of KSLW in loaded in saline and Pluronic solution. Minimum inhibitory concentration is the most commonly used pharmacodynamic parameter for the evaluation of efficacy, *in vitro* dose, and selection of anti infective agents (Davis and Bouzari, 2004). Using 96 well microtitre plates, optical density readings of treatment and control wells were compared with wells containing *S. epidermidis* in 1X Mueller Hinton broth (bacteria control). As presented in Fig. 2, KSLW retained its antibacterial activity when prepared in sterile water, saline, or Pluronic F68 block copolymer.

3.4.1. Sterile water

In sterile water KSLW significantly ($p < 0.001$) inhibited bacterial growth at concentrations $\geqslant$12.5 μg/ml (mean $OD_{600} = 0.104$), relative to bacteria control (mean $OD_{600} = 0.322$) (Fig. 2). Complete inhibition of growth was observed on blood agar plates at concentrations above 25 μg/ml. Hence, the MBC of KSLW in sterile water toward *S. epidermidis* equals 25 μg/ml, and the MIC equals 12.5 μg/ml.

3.4.2. F68

KSLW in Pluronic F68 block copolymer significantly ($p < 0.001$) inhibited growth of *S. epidermidis* compared to F68 alone, at peptide concentrations of $\geqslant$31.3 μg/ml, as determined by OD readings (Fig. 2). KSLW prepared in F68 resulted in complete growth inhibition of *S. epidermidis* on blood agar plates at a concentration of 62.5 μg/ml. Therefore, the MBC of KSLW in F68 equals 62.5 μg/ml, with a MIC of 31.3 μg/ml.

3.4.3. Saline

KSLW in saline significantly ($p < 0.001$) inhibited growth of *S. epidermidis* compared to saline alone, when KSLW concentrations were 31.3 μg/ml and greater, as determined by OD readings (Fig. 2). In addition, the KSLW saline preparation inhibited all growth on blood agar plates when KSLW at concentrations $\geqslant$31.3 μg/ml. Thus, the MBC/MIC of KSLW in saline toward *S. epidermidis* is 31.3 μg/ml. MICs and MBCs for KSLW in the tested delivery formats are listed in Table 2.

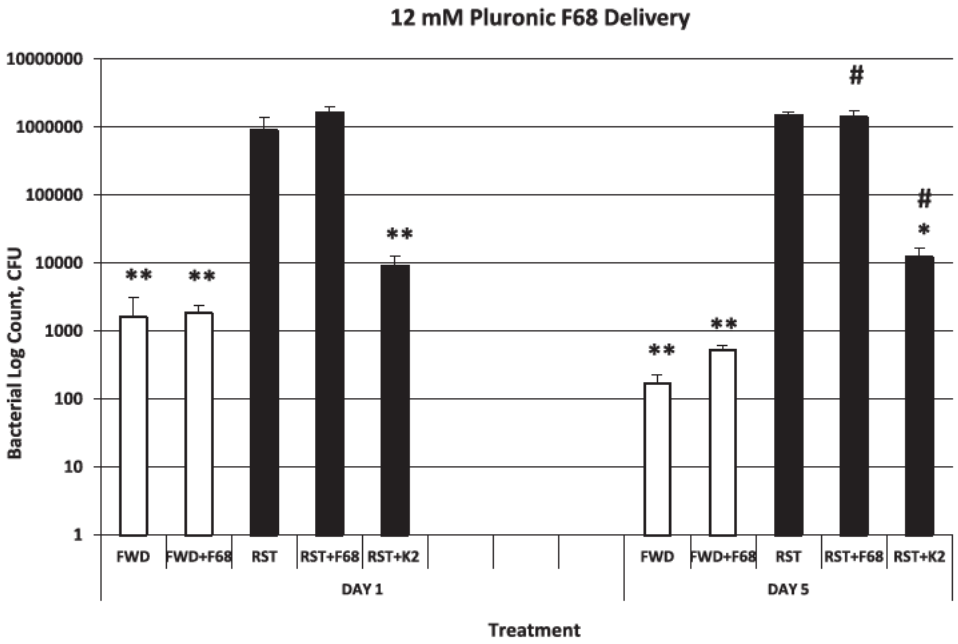

Fig. 1. Intradermal treatment with KSLW in F68 (days 1 and 5 post wounding). Day 1: restraint-stressed mice treated via intradermal injection of either 1 mg/ml KSLW (K1) or 2 mg/ml KSLW (K2), in F68 solution, demonstrated a significant decrease in bacterial load on day 1 post-wounding, compared to other RST mice. In addition, no significant differences ($p = 0.77$) were observed between RST mice treated with K2 and FWD control animals, at this early time point. Stressed mice treated with empty F68 (without peptide) displayed no differences from untreated stressed mice. Day 5: the antimicrobial effect of KSLW (K2) delivered in F68 block copolymer was sustained through day 5 post-wounding, with a greater than 2-log ($p < 0.01$) reduction in bacterial load among stressed mice. However, the level of reduction did not approach the levels observed in FWD control animals. There was a significant difference ($p = 0.028$) in wound bacteria counts from RST mice treated with K2, compared with FWD control mice. Stressed mice treated with empty F68 (without peptide) displayed no differences from RST mice on day 5. The data represent the means ± SEM; $n = 15$ mice per group (day 1) in three independent experiments. $n = 10$ mice per group (day 5) in two independent experiments. $^{*}p < 0.05$ as compared to RST. $^{**}p < 0.01$ as compared to RST. $^{\#}p < 0.03$ as compared to control (FWD). K2 = KSLW at 2 mg/ml (in Pluronic F68 block copolymer).

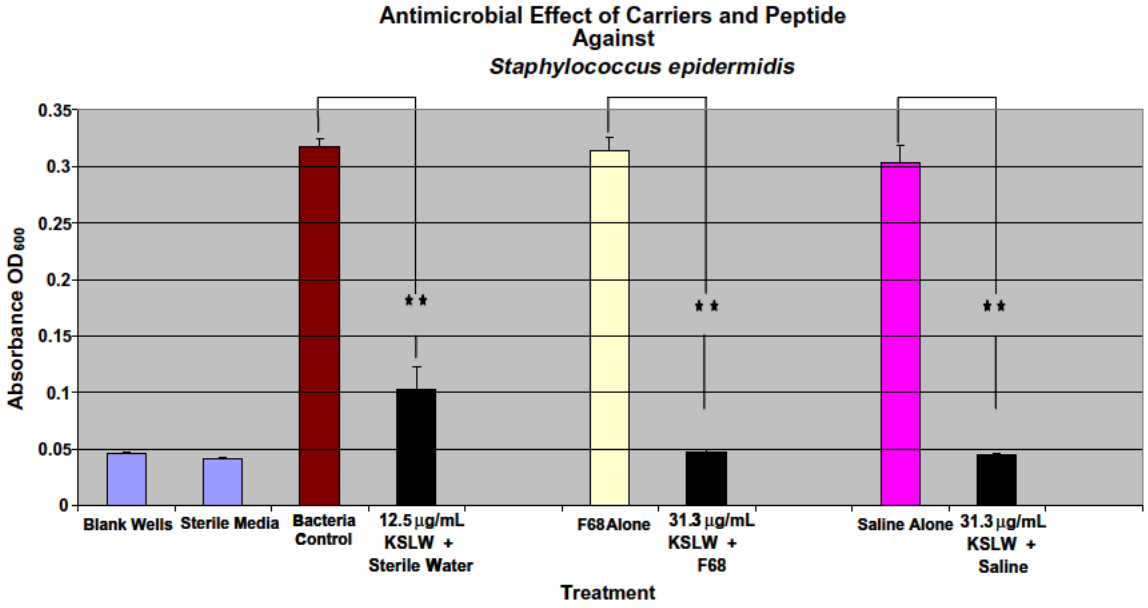

Fig. 2. Antimicrobial activity of KSLW against *S. epidermidis* in different carriers (OD_{600} readings). In sterile water, KSLW significantly inhibited bacterial growth at concentrations ⩾12.5 μg/ml, relative to the bacteria control (grown in 1X Mueller Hinton Broth). In saline, KSLW significantly inhibited bacterial growth compared to saline alone, at concentrations ⩾31.3 μg/ml. At concentrations ⩾31.3 μg/ml, KSLW loaded in F68 significantly inhibited bacterial growth, compared to F68 alone. Microbial growth levels observed following treatment with either "empty" carrier preparation were comparable to the bacteria control. $^{**}p < 0.001$ as compared to carriers loaded with KSLW. All assays were completed in triplicate.

3.5. KSLW release kinetics

HiLyteFluor™488 labeled KSLW loaded into 12 mM Pluronic F68 at a concentration of 2 mg/mL. Hourly spectrofluorometric measurements were begun after 1 h of dialysis. A sustained release of KSLW was determined at 50% per hour. The release of KSLW plateaued after 8 h, with no detectable decreases in fluo rescent peptide concentration observed through 15 h (Fig. 3). The data represent two independent experiments conducted in triplicate.

Table 2
MIC/MBC against *Staphylococcus epidermidis*.

KSLW minimum inhibitory and minimum bactericidal concentrations		
Peptide and carrier	MIC (μg/mL)	MBC (μg/mL)
KSLW + 12 mM F68	31.3	62.5
KSLW + Saline	31.3	31.3

Minimum inhibitory and bactericidal concentrations were determined for KSLW against *S. epidermidis* in various carriers. *In vitro* assays were performed via OD_{600} readings conducted on 96-well microtitre plates and plating on 5% Sheep blood agar. Experiments were completed at least two times in triplicate.

3.6. Wound closure

We evaluated changes in wound area in control (FWD) and stressed mice groups, as indices of wound closure. Healing rates among FWD mice were significantly ($p < 0.05$) faster than un treated RST and RST mice treated with F68 alone from days 2 to 5, and RST mice treated with KSLW from days 3 through five post wounding (Fig. 4). Treatment of RST mice with KSLW (deliv ered in F68) improved healing, resulting in significantly faster ($p < 0.05$) wound closure from days 2 to 5 post wounding, com pared to untreated RST mice and RST mice treated with F68 alone (Fig. 4). No differences were observed between with FWD mice and FWD mice treated with F68.

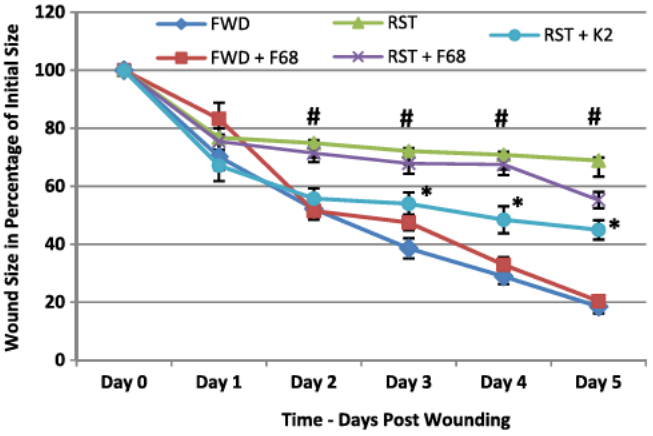

Fig. 4. Wound closure over time. Healing rates among FWD mice were significantly ($p < 0.05$) faster than untreated RST and RST mice treated with F68 alone from days 2 to 5, and RST mice treated with KSLW from days 3 through 5 post-wounding. Treatment of RST mice with KSLW (delivered in F68) improved healing, resulting in significantly faster ($p < 0.05$) wound closure from days 2 to 5 post-wounding, compared to untreated RST mice and RST mice treated with F68 alone. No differences were observed between with FWD mice and FWD mice treated with F68. Values represent the means ± SEM. $n = 10$ mice per group per day. $^{\#}p < 0.05$ for both RST and RST + F68, as compared to RST + K2. $^{*}p < 0.05$ for RST + K2, as compared to FWD. K2 = KSLW at 2 mg/ml (in 12 mM Pluronic F68 block copolymer).

3.7. TLR 4 mRNA expression

TLR 4 expression was quantified at the mRNA level, via real time RT PCR, on day 5 post wounding. All RST groups demon strated significantly lower levels of TLR4 mRNA on day 5 post wounding, compared with FWD (Fig. 5). Treatment with KSLW significantly increased TLR4 mRNA in stressed mice (Fig. 5). No differences were observed between untreated stressed mice and stressed mice treated with the F68 carrier alone.

4. Discussion

Dysregulated inflammatory responses may result in increased risk of microbial infection and impaired wound healing. It is well accepted that psychological stress suppresses immune/inflamma tory responses to microbial components (Dhabhar and McEwen, 1999). Previous studies in our lab have demonstrated increased susceptibility to opportunistic bacterial infection and increased bacterial counts in wounds of stressed animals (Rojas et al., 2002). Additionally, psychological stress has been shown to delay wound closure (Padgett et al., 1998) and wound contraction (Horan et al., 2005), and alter proinflammatory cytokine expression (Mercado et al., 2002). Experimental studies have shown that a level of bacterial growth greater than 10^5 organisms per gram of tissue, which compromises the host bacteria equilibrium, is necessary to cause wound infection (Robson, 1997). Bacteria are thought to play a critical role in delayed healing by altering host cell function, and lowering the level of endogenous growth factors (Robson et al., 1999). Strategies to control the risk of infection, and the level of bacterial activity, are generally directed toward several variables number of bacteria, strength of their virulence, and the immune status of the host (Robson, 1997).

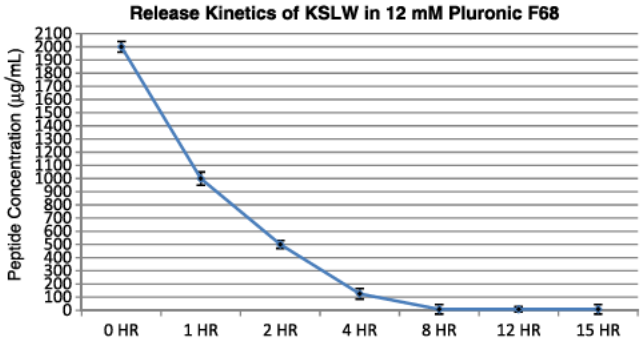

Fig. 3. KSLW release kinetics 15 h. HiLyteFluor™488-labeled KSLW loaded into 12 mM Pluronic F68 at a concentration of 2 mg/mL. Hourly spectrofluorometric measurements were begun after 1 h of dialysis. A sustained release of KSLW was determined at 50% per hour. The release of KSLW plateaued after 8 h, with no detectable decreases in fluorescent peptide concentration observed through 15 h. These data represent two independent experiments conducted in triplicate.

In this study, we tested the hypothesis that the synthetic deca peptide KSLW enhances bacterial clearance during stress impaired healing. Various techniques were compared to determine an effec tive route for drug administration, and to establish an optimal car rier system for improving peptide delivery to the wounds of stressed mice. A significant reduction in wound bacteria was ob served in stressed mice treated with topically administered KSLW in saline, but the effect was not sustained beyond 1 day post wounding. The route of delivery was modified to introduce KSLW intradermally. The antimicrobial effect was not observed beyond 2 days post wounding (data not shown), when delivered in saline solution. We then used the same single dose intradermal injection technique, to administer KSLW, loaded in Pluronic F68 block copolymer, to the wound site. Treatment with the KSLW + F68 solution demonstrated a positive effect in reducing bacterial load in the wounds of stressed animals, over time. Significant differ ences were observed on days 1 and 5, relative to untreated RST mice and RST mice treated with empty carrier. In addition, analysis of gene expression in the excisional wounds, on day 5, demon strated increases in TLR 4 mRNA, among RST mice treated intra dermally with KSLW + F68, when compared to untreated RST mice and RST mice injected with F68 alone.

Our findings, using a single application of KSLW dissolved in saline, demonstrated a significant, but limited, antimicrobial effect. Bacterial load in the wounds of RST mice was reduced, with topical KSLW, on day 1 post wounding. However, no differences were ob served beyond this early time point, which suggested that the stability of the peptide, as well as its retention in the wound, were

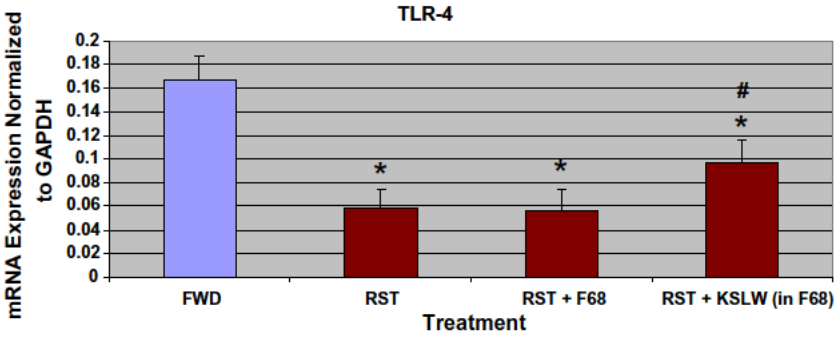

Fig. 5. TLR-4 mRNA expression (day 5). All RST groups demonstrated significant reductions in TLR4 mRNA on day 5 post-wounding, compared with FWD. Treatment with KSLW significantly increased TLR4 mRNA in RST mice. No significant differences were observed between untreated RST and RST mice treated with carrier alone. These data suggest that macrophage activation and wound debridement, may be enhanced in stressed mice treated with the antimicrobial peptide. $n = 4$ mice per group. $^{*}p < 0.03$ as compared with control (FWD). $^{\#}p < 0.05$ as compared with both untreated RST and RST treated with F68 alone. The data represent the means ± SEM.

compromised by this route of delivery. A plethora of proteolytic molecules, such as serine proteases, is released from stimulated leukocytes during inflammation (Doumas et al., 2005) and injury. Proteinases are involved in a wide variety of biological processes (Hiemstra, 2002). Tissue proteinases, including kallikeins and kal likrein related peptidases (Stefansson et al., 2006, 2008; Borgoño et al., 2007), urokinase type plasminogen activator (Yager and Nwomeh, 1999), TNF α induced matrix metalloproteinases (Trengove et al., 1999; Ito et al., 1990), and leukocyte derived pro teases, such as elastase, cathepsin G and proteinase 3 (Barrick et al., 1999; Yager and Nwomeh, 1999), have the capacity to degrade numerous connective tissue macromolecules, and could poten tially interfere with the stability of small exogenous peptide molecules, in a nonselective manner. In addition to known bacteria derived proteases with definitive roles (Qin et al., 2000; Wang et al., 2007), many bacteria express a variety of proteases, with nonspecific and powerful enzymatic activity, which degrade pro teins involved in innate immunity (Potempa and Pike, 2009).

Hydrophobic interactions of the propylene oxide (PO) blocks form the driving force behind Pluronic micellization, in which PO blocks self assemble into the inner core of the micelles covered by the hydrophilic corona from ethylene oxide (EO) blocks (Kaba nov et al., 2002). This core shell structure is critical for their utility in drug delivery applications (Kabanov et al., 1995, 2002). Various micellar morphologies, including lamella and rods, can form during micellization, Pluronic micelles are commonly thought to exist as spherical entities, which is correct for most block copolymers with an EO content above 30% (Kabanov et al., 2002), and commonly have an average hydrodynamic diameter between 20 and 80 nm. In addition to factors such as the length of the both the PO and EO blocks, temperature, ionic strength, and pH of the media, all of which influence micelle assembly, the critical micellar concen tration (CMC) is paramount for micellization. The CMC determines the stability of micelles against possible dilution in body fluids, and determines the maximal achievable concentration of Pluronic uni mers, to which cells will be exposed, thereby defining the biologi cal effects, which Pluronic itself (Kabanov et al., 2002) as well as delivered drugs or peptides, will exert on cells. In block copoly mers, the hydrophobic cores of the micelles can be loaded with small hydrophobic drugs, either by physically loading the drug, or by conjugating it to the amino acid pendant acid groups (Hoff man, 2008). Drug molecules with intermediate hydrophobic/ hydrophilic ratios have intermediate positions within the micelle particle (Torchilin, 2001). In this study, Pluronic F68 was selected as an *in vivo* drug delivery system for KSLW based on its capacity to self assemble into micelles above a low CMC, and incorporate therapeutic agents (Hoffman, 2008). Stressed mice treated with empty Pluronic F68 carrier displayed no differences in bacterial load, on days 1 or 5 post wounding, when compared to untreated stressed mice. Treatment with 2 mg/ml KSLW delivered in Pluronic F68 resulted in a sustained antimicrobial effect through day 5, with a 2 log ($p < 0.01$) reduction in bacterial load among stressed mice. However, the demonstrated amount of bacterial reduction in KSLW treated stressed mice did not approach the levels observed among control mice. These findings suggest that F68 is an efficient carrier for KSLW, which improves its stability in the wound envi ronment. Key to the selection of the micelle forming Pluronic F68, was not only protecting peptide stability but enhancing its substantivity. We hypothesize that the physical changes of this poloxamer at the optimized molar concentration, combined with a therapeutic peptide concentration, resulted in the retention of KSLW at the wound site, which may have been diluted during top ical and intradermal administration in saline. The expected reten tion in the tissue supports increased direct antimicrobial function and indirect cellular activity during early inflammation.

We examined the release kinetics of KSLW in F68 solution at a concentration of 2 mg/ml, starting 1 h post loading. The release a drugs from micelles is thought to occur through several processes: diffusion, micelle degradation, and ion displacement (Kabanov and Vinogradov, 2008). In this study, the release rate of KSLW from Plu ronic F68 occurred at approximately 50% per hour, which then plateaued at 8 h, indicating a limited sustained release effect. Sus tained release means that the drug will be released under first or der kinetics [constant proportion (e.g., 10%) per unit time] compared to controlled release in which drugs are released under zero order kinetics [constant mass (e.g., 10 mg) per unit time] (Hoffman, 2008). However, these *in vitro* experiments, with KSLW, offer limited useful information. We were not able to quantify the amount of fluorescent peptide actually loaded into micelles, to give a micelle peptide ratio, and the experimental conditions were void of physiologic environmental factors such as fluctuating pH in the wound, protease activity, micellar structural alterations with phys iologic temperature, and possible effects of F68 Pluronic solution on host cells.

Concurrent with the waning of the inflammatory phase of heal ing is the formation of granulation tissue and epithelialization, which are primarily observed during the proliferative phase, begin ning approximately 4 days following injury. Injury disrupts the normal skin barrier, and thus, wound healing depends on the abil ity to clear foreign material and resist infection (Hopf and Rollins, 2007). Bacterial products are known to directly inhibit keratino cyte migration in persistent wounds (Loryman and Mansbridge, 2008). Previous studies from our group have shown that mice sub jected to restraint stress healed an average of 30% more slowly than control mice (Padgett et al., 1998) and demonstrated increased susceptibility to bacterial infection (Rojas et al., 2002).

Here, we determined the effect of KSLW on wound closure, when delivered in a nanocarrier system. The significantly faster rates of closure, in wounds of stressed mice treated with KSLW, suggests that the peptide regulates inflammation, perhaps via removal of microbial components through direct killing and cellular clearance of bacteria and debris. Therefore, we propose that the expected bioactivity of the peptide (on bacteria as well as the host), along with the improved substantivity, of the Pluronic delivery format, resulted in a heightened inflammatory response during early healing. With amelioration of psychological stress associated delayed healing patterns, a normal wound healing trajectory and faster closure may be observed.

Toll like receptors are pattern recognition receptors that may be found on a variety of cells, and are particularly important on monocytes, macrophages and dendritic cells (Bowie and O'Neill, 2000; Huang et al., 2007). Wu et al. (2009) demonstrated a role for TLR 4 in the LPS activation of cultured macrophages, using RNAi knock down methods. Gene deletion studies further demonstrated that TLR 4 is principally responsible for LPS induced activation (Huang et al., 2007) of macrophages through NF κB (Andreakos et al., 2005; Huang et al., 2007) and MAP kinase (Takeda and Akira, 2005; Huang et al., 2007) signaling pathways. Previous work in our lab, suggests that stress down regulates the expression of molecules involved in TLR 4 signaling, particularly TLR 4 related microRNAs, which could compromise the inflammatory response and bacterial clearance in wounds of stressed mice Tymen et al., 2011. This study evaluated the effect, of KSLW, on host cell function by measuring gene expression of TLR 4, which is expressed on macrophages and signals the presence of LPS, by associating with CD14 (Poltorak et al., 1998). Studies by Rojas et al. (2002) showed no differences in the number of macrophages from day 5 wounds of FWD and RST mice, which suggested possible impairment of macrophage function in RST mice (Mercado et al., 2002). The quantification of TLR 4 induction, at day 5, served as an indicator for macrophage activation and wound debridement. Macrophage migration to the wound site generally occurs between 48 and 96 h post wounding (Park and Barbul, 2004). Immunohistochemical analyses of murine excisional wounds by Mori et al. (2002), showed macrophage recruitment to be most evident at 6 days after injury. Our results demonstrated a 2.7 fold reduction in TLR 4 mRNA expression among untreated RST mice, compared with FWD mice. Treatment of RST mice with KSLW, resulted in a significant increase in TLR 4 mRNA levels on day 5. The demonstrated effect of KSLW on TLR 4 expression suggests a possible role in macrophage function and activation beyond its inherent antimicrobial activity. However, the effect of KSLW on TLR 4 upregulation, and macrophage activity, may be influenced by decreased bacterial activity in the wound, due to early treatment with the antimicrobial agent. Inherently, direct bacterial killing would result in an increased number of macrophages, recruited to clean up the wound site (effete neutrophils and lysed bacteria) and upregulation of TLR 4. Earlier investigations demonstrated that KSLW is chemotactic for neutrophils *in vitro* (Williams et al., 2012). So, an expected observation of macrophage chemotaxis, in response to KSLW, with a resultant increase in wound macrophage counts is plausible. Furthermore, there is a possibility of a direct effect on TLR expression in macrophages, which may function to ameliorate the dysregulated inflammatory response in the wounds of stressed animals. Ongoing studies with collaborators are underway to investigate KSLW's bioactivities at the molecular level and its effect in various skin cell types and other mammalian cell lines.

5. Conclusion

This work demonstrated that KSLW, when delivered in a triblock copolymer solution, enhances bacterial clearance in restraint stressed mice and thus improves the potential for successful wound healing. The release of KSLW from the Pluronic micelles appears to be based on first order kinetics. However, the actual metabolism and half life of the micelles, in the wound environment, have not been determined. Further investigations will allow us to determine the specific influences of micelle loaded KSLW on host cell functions, and characterize the peptide as a multifunctional molecule.

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